(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 527 283 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
26.11.1997 Bulletin 1997/48

(51) Int. Cl.[6]: **A23L 1/305**, A61K 35/20, A61K 38/18

(21) Application number: **91810629.5**

(22) Date of filing: **12.08.1991**

(54) Food composition

Nahrungsmittelzusammensetzung

Composition alimentaire

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(43) Date of publication of application:
17.02.1993 Bulletin 1993/07

(73) Proprietor:
SOCIETE DES PRODUITS NESTLE S.A.
1800 Vevey (CH)

(72) Inventors:
- **Huggett, Anthony C.**
  CH-1012 Lausanne (CH)
- **Hughes, Anne**
  CH-1806 Saint Legier (CH)
- **Schiffrin, Edwardo**
  CH-1023 Crissier (CH)

(56) References cited:
EP-A- 0 269 408
EP-A- 0 313 515
WO-A-90/00900
WO-A-90/03812
US-A- 4 440 860

- **EUROPEAN JOURNAL OF BIOCHEMISTRY vol. 197, no. 2, April 1991, BERLIN, G pages 353 - 358; D.A.COX ET AL.: 'Isolation and characterization of milk growth factor, a transforming-growth-factor-beta2-related polypeptide, from bovine milk'**
- **IMMUNOLOGY vol. 72, no. 2, February 1991, OXFORD, GB pages 491 - 496; A. HUGHES ET AL: 'Expression of MHC class II (Ia) antigen by the neonatal enterocyte: the effect of treatment with interferon-gamma'**
- **CHEMICAL ABSTRACTS, vol. 107, no. 9, 31 August 1987, Columbus, Ohio, US; abstract no. 76199V, F. H. MORRISS JR.: 'Growth factors in milk' page 570 ;column 2 ; & Hum. Milk Infant Nutr. Health 1986, 98-114**
- **PEDIATRIC RESEARCH vol. 25, no. 4, April 1989, BALTIMORE, US page 269A; M. EL-YOUSSEF ET AL.: 'Identification of tumor necrosis factor alpha (TNF-alpha) and transforming growth factor beta (TGF-beta) in murine milk'**
- **JOURNAL OF PROTEIN CHEMISTRY vol. 10, no. 5, October 1991, NEW YORK, US pages 565 - 575; Y. JIN ET AL.: 'Separation, purification, and sequence identification of TGF-beta1 and TGF-beta2 from bovine milk'**

Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

EP 0 527 283 B1

Printed by Rank Xerox (UK) Business Services
2.14.23/3.4

Description

Field of the Invention

This invention relates to the use of milk-derived polypeptides of the transforming growth factor beta family for the regulation of immune responses at the gut level associated with MHC (major histocompatibility complex).

This invention relates especially to the use of mammalian milk or colostrum derived TGF-β-like MGF (milk growth factor) for the preparation of a food composition, an enteral preparation or a pharmaceutical composition, as well as to a food composition or to an enteral preparation containing an effective amount of mammalian milk or colostrum derived TGF-β-like MGF.

Background of the Invention and Prior Art

Human and bovine milk contain many biologically active polypeptides including growth factors (West, D.W. Exp.Clin.Endocrinol. 8 145-146,1989). One of these factors, MGF (milk growth factor) was recently identified as identical to or having close homology to a member of the transforming growth factor beta (TGF-β) family, notably TGF-β2 (Cox D.A. et al. Eur. J. Biochem. 197 353-358, 1991). TGF-β is the general name for a family of polypeptides consisting of at least 5 distinct but closely related members, which have considerable structural and biological homologies (Roberts, A.B., et al. In: Peptide Growth Factors and their Receptors Vol. 1, pp. 419-472, Eds. Sporn M.B. et al., Springer, 1990). TGF-βs are homodimeric proteins of about 25 kDa consisting of identical 12.5 kDa polypeptide chains linked through disulphide bridges. They may form latent complexes with other proteins and these complexes may be activated by acid treatment or mild proteolysis (Roberts, A.B. et al.). They are multipotent, having a number of biological activities depending upon the target cell type, its state of differentiation and the presence of other factors. These activities include stimulation or inhibition of cell proliferation and differentiation, regulation of extracellular matrix deposition, immunomodulation, steroidogenesis and angiogenesis (Roberts, A.B. et al.).

Expression of MHC-Class II on the surface of antigen-presenting cells is a prerequisite for the presentation of exogenous antigen to T-cells (Benacerraf, B., Science 212 1229, 1981). Epithelial cells in the intestinal villus of the adult rodent constitutively express MHC-Class II while its expression by crypt cells depends in part on their spatial location in the intestine (Hughes, A., et al. Immunol. 72 491, 1991). In the postpartum period in the rodent there is little or no expression of MHC-Class II by enterocytes until after weaning, thus indicating the presence of a suppressive factor in milk (Hughes, A. et al.).

TGF-βs, including TGF-β2, have a number of immunoregulatory properties and act at several stages of the inflammatory and immune reaction. For example they inhibit the proliferation of T and B lymphocytes (Kerhl, J.H., et al. J.Immunol. 137:3855-3860, 1986; Kerhl, J.H., et al. J.Exp.Med. 163:1037-1050, 1986) and thymocytes (Ristow, H.J. Proc.Natl.Acad.Sci.USA 83 5531-5534,1986). They also antagonize the effects of interleukins including IL-1, IL-2 and IL-3 and other immunoregulatory agents such as tumor necrosis factor and interferons (Roberts, A.B. et al.). Although most of their effects on immune cells are inhibitory, TGF-βs appear to play a critical role in isotype switching of IgG and IgM secreting cells to IgA secreting cells (Lebman, D.A., et al. J.Immunol. 144:952-959, 1990). With particular reference to reported immunosuppressive effects of MGF, this factor has been shown to decrease the proliferation of human lymphocytes induced by anti-CD3 or interleukins (Stoeck, M., et al. FEBS Lett. 249 289-292,1989); Stoeck, M., et al. J.Immunol. 143 3258-3265, 1989). TGF-βs interfere with certain accessory cell functions important in antigen presentation and specifically were shown to suppress MHC-Class II expression by melanomas, glial cells and astrocytes (Czarniecki, C.W., et al J.Immunol. 140 4217-4223, 1988; Schlusener H.J. J.Neuroimmunol. 24 41-47, 1990; Zuber, P. et al. Eur.J.Immunol. 18 1623-1626,1988). However, the regulation of MHC-Class II expression on epithelial cells in the intestine by TGF-βs or MGF has not hitherto been reported.

Altered regulation of MHC-Class II has been implicated in several gastrointestinal disorders. The presence of active inflammation at the gut level generally results in an increase in MHC-Class II expression on human intestinal epithelium and lamina propria (Mayer, L., et al. Gastroenterology 100 3-12, 1991). This increase is a conspicuous component of Inflammatory Bowel Disease (IBD), (Mayer, L. et al.). In IBD, tissue damage is due either to an autoimmune attack on the cellular components of the host intestinal mucosa (Snook, J.A., et al. Gut 32 163-166, 1991), or to a disorder in the mucosal immune regulation with an over-reactivity to luminal antigens in the gut, based on a defective down-regulation of this response (Challenges in IBD Research: Agenda for the 1990's. National Foundation for Ileitis and Colitis. Feb. 21, 1990. Washington D.C.).

Both possibilities imply the existence of a disregulation of the mucosal immune response and emphasize an immunologic role in the initiation and perpetuation of the inflammatory response.

PEDIATRIC RESEARCH, vol. 25, no.4, April 1989, BALTIMORE, US, page 269A, M. EL-YOUSSEF et al., disclose that TGF-alpha and TGF-β are present in murine milk and say that these two factors, known to modulate cell growth and immune function, may thereby influence the maturation of the gastrointestinal mucosal barrier in the breast-fed neonate.

EP-A-0313515 discloses a Milk Growth Factor (MGF) and a process for isolating it from fresh bovine milk, the use of this MGF for the promotion of surface wound healing or healing of internal wound, the use of immunosuppressively effective amounts of this MGF for preventing inflammations and the use of growth promoting amounts of this MGF for stimulating growth of a mammal.

European Journal of Biochemistry, vol. 197, no 2, April 1991, pp 353-358, discloses that the MGF of EP-A-0313515 has a close similarity with TGF-β2 and mentions its immunosuppressive effects and its ability to promote wound healing responses.

Journal of Protein Chemistry, vol. 10, no 5, 1991, pp 565-575 establishes that the major component of the MGF of EP-A-0313515 is TGF-β2.

EP-A-0269408 discloses the use of TGF-β in the preparation of a medicament for treating an inflammatory disorder accompanied by a Class II or Class III immune response, such as rheumatoid arthritis, systemic lupus erythematosus or inflammatory bowel disease.

WO 90/00900 discloses a method of treating an inflammatory disorder such as adult respiratory distress syndrome, rheumatoid arthritis, asthma, emphysema, acute glomerular nephritis, inflammatory bowel disease, bowelpulmonary fibrosis and sarcoidosis, comprising a step of administering to a patient a therapeutically effective amount of TGF-β.

Immunology, vol. 5, no 2, Feb. 1991, pp. 491-496, says that because MHC class II antigen is present in lamina propria cells but not in enterocytes during nursing, it is possible that breast milk contains an inhibitor of enterocyte MHC class II expression, and that milk components such as prostaglandin and cortisol, as well as TGF-β, are known inhibitors of MHC class II expression.

WO 90/03812 relates the fact that TGF-β has been found to suppress the expression of MHC class II on human cells induced by human interferon and discloses a method for the treatment of grafts prior to transplantation , by incubating, coating or perfusing them with TGF-β.

Object of the Invention

The object of the present invention is to provide a food composition or an oral and/or enteral preparation for regulating MHC mediated immune responses in the mammalian gastrointestinal tract, and more especially for the treatment of Inflammatory Bowel Diseases (e.g. Crohn's disease, Ulcerative Colitis) or Graft-vs-Host reactions in humans or animals, for the prevention of diarrhea in weaning humans or animals, or for the prevention of allergic reactions in the gastrointestinal tract in humans or animals.

Summary of the Invention

The present invention comprises the use of a transforming growth factor-beta2 (TGF-β2) containing acid casein fraction isolated from bovine milk for the preparation of a food composition or of an oral and/or enteral preparation for the modulation of major histocompatibility complex-Class II (MHC-Class II) expression by intestinal epithelial cells in humans or animals, wherein the food composition or the oral and/or enteral preparation contains 0.1 to 50 μg TGF-β2 per g of dry matter, preferably for the treatment of Inflammatory Bowel Diseases, especially the Crohn's disease, or Graft-vs-Host reactions in humans or animals, or for the prevention of diarrhea in weaning humans or animals, or for the prevention of allergic reactions in the gastrointestinal tract in humans or animals.

Detailed Disclosure of the Invention

For preparing the food composition or the enteral preparation, or for carrying out the uses according to the present invention, a bioactive milk component, identical to or with close homology to TGF-β2 may be prepared in an enriched form from mammalian milk products, especially from bovine milk products, e.g. as disclosed in EP-A1-313515 (CIBA-GEIGY AG) p. 6 l. 11 to p. 7 l. 34 and Examples 1 to 3, and having TGF-β2-like activity on the proliferation of mammalian liver epithelial cells and on the expression of MHC by mammalian intestinal epithelial cells. Henceforth this bioactive milk factor is termed TGF-β2-like MGF.

Test 1.TGF-βs in Milks

Normal rat liver epithelial (RLE) cells which have previously been shown to be sensitive to the growth inhibitory effects of TGF-βs (Huggett, A.C., et al. Cancer Res. 50 7468-7475, 1990) were incorporated into a bioassay for the analysis of TGF-βs in milks and in acid-treated milk fractions and milk powders. Measurement of inhibition of DNA synthesis by ^{3}H-Thymidine incorporation was performed as described previously (HuggettA.C. et al.). Antibodies raised against TGF-βs (British Bio-technology Ltd.) were coincubated with standards or samples prior to bioassay analysis in order to determine inhibitory activity specific to TGF-β isoforms. Using this assay a 50% inhibition of RLE cell DNA synthesis is obtained with 50 pg/ml of TGF-β1 or TGF-β2.

Human and bovine milk were delipidated by centrifugation, desalted on PD-10 columns (Pharmacia) eluted with PBS and then sterilized by filtration through 0.2μm membranes (Millipore). Protein contents were monitored using the method of Smith et al (Smith P.K., et al. Anal. Biochem. 150: 76-85, 1985). For analysis of latent acid-activatable TGF-βs, the milk samples were adjusted to pH 4 with 1N HCl, centrifuged at 40000 g for 60 min to separate whey and casein fractions which were then neutralized with 1N NaOH and dialyzed against PBS. Dilutions were then analyzed using the RLE cell bioassay together with a series of TGF-β standard solutions. An estimation of the amount of TGF-β-like activity was determined by a comparison of the degree of inhibition of DNA synthesis obtained with the samples against TGF-β standard curves. The identification of specific isoforms of TGF-β was determined by examining the effects of isoform-specific neutralizing antibodies on the inhibitory activity.

This test demonstrates that both human and cows milk contain acid-activatable TGF-β2-like MGF which is mainly associated with the casein fraction (Table 1).

Table 1

TGF-β2-like MGF activity in Milks	
Sample	Active TGF-β2-like MGF (μg/g protein)
Bovine Milk	< 0.01
Bovine Acid Casein	0.52
Human Milk*	< 0.2
Human Acid Casein	0.75

*This value is overestimated due to the large amounts of EGF in these samples which interfere with the assay.

Tests 2 and 3

Suppression of MHC-Class II Expression by Intestinal Epithelial Cells

The HT-29 intestinal epithelial line derived from human colonic epithelial cells (Fogh, J. et al. In: Human Tumor Cells "in vitro". J.Fogh, ed. Plenum Publishing Corp., New York, pp. 115, 1975), were maintained in an undifferentiated state in glucose-containing media (Zweibaum, A., et al. J.Cell.Physiol., 122: 21, 1985). When the cells reached 70-80% confluence, they were exposed, over a 48h period, to one of the following treatments:- human recombinant interferon-gamma (IFN-γ, 100 U/ml) alone (Boehringer Mannheim); IFN-γ in combination with TGF-β2; IFN-γ followed by TGF-β2; TGF-β2 alone followed by IFN-γ; or, as a control, culture media alone. Cells were washed and retreated after the first 24h. TGF-β2 was used at doses ranging from 0.05ng to 4ng per ml. Following the treatment period, the cells were washed, fixed and the plates stored frozen at -20°C until required.

The avidin-biotin complex method of immunoperoxidase staining (Cerf-Bensussan, N., et al. J.Immunol., 130: 2615, 1983) was performed on monolayers utilising the mouse monoclonal antibody L234 (Becton Dickinson), which recognises the human MHC-Class II histocompatibility antigen HLA-DR. Mouse myeloma IgG protein (Zymed) served as a control. In another series of experiments, a normal rat small intestinal cell line, IEC-18 (Quaroni, A., et al. J.Cell Biology, 80 248, 1979) was grown to 50% confluency and subjected to IFN-γ and/or TGF-β2 in the combinations listed above. Cells were then detached from the culture dishes using Versene (Life Technologies Ltd.) and stained, in suspension, using a standard, direct immunofluorescence technique. Briefly, cells were washed, incubated with normal serum for 5min and then with the FITC-conjugated mouse monoclonal antibody MRC OX-6 (Serotec) which recognises the rat Class II MHC antigen. Cells were then washed and fixed for at least 1h with 1% paraformaldehyde before analysis in the FACScan (Becton Dickinson).

During food allergy and inflammatory diseases, intestinal epithelial cells express high levels of Class II antigen thought to be mediated, at least in part, by inflammatory cytokines such as IFN-γ. The HT-29 undifferentiated cells employed in the assay described, do not constitutively express Class II molecules. To partially mimic events taking place during the onset of intestinal inflammation, the cells were exposed to IFN-γ. The effect of TGF-β2 on this reaction was then examined. Exposure to IFN-γ induced Class II expression on the HT-29 cells but this effect was abrogated by pretreatment with TGF-β2 at all the doses tested (Table 2). In contrast, the other combinations of cytokines tested resulted in high levels of Class II expression. The majority of IEC-18 cells already expressed Class II molecules but

showed increased expression following treatment with IFN-γ (Table 3). Once again, TGF-β2 suppressed this induction. Thus, at the onset of inflammatory intestinal reactions, TGF-β2 may modulate local expression of Class II antigens.

Table 2

Effect of TGF-β2 on MHC-Class II expression by human intestinal epithelial cells (HT-29).		
Treatment		MHC-II Expression
(0-24h)	(24-48h)	
none	none	-
none	IFN-γ	++
IFN-	none	++
IFN-γ	IFN-γ	+++
TGF-β2	none	-
TGF-β2	TGF-β2	-
TGF-β2	IFN-γ	-
IFN-γ	TGF-β2	++
TGF-β2+IFN-γ	TGF-β2+IFN-γ	++
Staining: - negative + weak ++ strong +++ very strong		

Table 3

Effect of TGF-β2 on MHC-Class II expression by rat intestinal epithelial cells (IEC-18).		
Treatment		MHC-II Expression (% positive cells)
(0-24h)	(24-48h)	
none	none	73.6 ± 1.5
none	IFN-γ	85.3 ± 5.3
IFN-γ	IFN-γ	95.8 ± 0.6
TGF-β2	none	67.3 ± 1.8
TGF-β2	IFN-γ	75.8 ± 0.3
TGF-β2+IFN-γ	TGF-β2+IFN-γ	86.9 ± 1.5

The demonstration of MCH-Class II antigens on human and rodent intestinal cells supports the notion that these cells may act as antigen presenting cells (Mayer, L., et al. J.Exp. Med. 166 1471-1483, 1987. The epithelial cell of the intestine has been considered a major participant in the etiopathogenesis of IBD. An increase in their expression of MHC-Class II could lead to an increased epithelial-T-helper lymphocyte interaction and this, in turn, could be a primary event in IBD or a perpetuating mechanism. The present studies demonstrate for the first time the action of TGF-β2 (and TGF-β2-like MGF) on suppression of MHC-Class II expression on intestinal epithelial cells. According to these findings, the availability of an immunosuppressive agent acting topically at the surface of the intestinal mucosa could provide a

new tool to interrupt the pathogenic mechanism involved in IBD and other inflammatory-immune conditions in the gut, namely Coeliac Disease and Graft-vs-Host reactions.

Example 1

TGF-β2-like MGF prepared in enriched form from bovine milk as disclosed above is added to a nutritionally balanced enteral product comprising about 10% of dry matter in such a quantity that the enteral preparation thus obtained comprises an amount of about 0.1 to 50, preferably 0.5 to 20 μg of TGF-β2-like MGF per g of dry matter.

The enteral preparations prepared in this way are effective in suppressing MHC-Class II expression by intestinal epithelial cells.

Example 2

TGF-β2-like MGF prepared in enriched form from bovine milk as disclosed above is added to a balanced food product in liquid or powder form in such a quantity that the food composition thus obtained comprises an amount of about 0.1 to 50, preferably 0.5 to 20 μg of TGF-β2-like MGF per g of dry matter.

The food composition prepared in this way are effective in suppressing MHC-Class II expression by intestinal epithelial cells.

Claims

1. Use of a transforming growth factor-beta2 (TGF-β2) containing acid casein fraction isolated from bovine milk for the preparation of a food composition or of an oral and/or enteral preparation for the modulation of major histocompatibility complex-Class II (MHC-Class II) expression by intestinal epithelial cells in humans or animals, wherein the food composition or the oral and/or enteral preparation contains 0.1 to 50 μg TGF-β2 per g of dry matter.

2. Use according to claim 1, wherein said food composition or oral and/or enteral preparation is for the treatment of Inflammatory Bowel Diseases or Graft-vs-Host reactions in humans or animals.

3. Use according to claim 2, wherein said Inflammatory Bowel Disease is the Crohn's disease.

4. Use according to claim 1, wherein said food composition or oral and/or enteral preparation is for the prevention of diarrhea in weaning humans or animals.

5. Use according to claim 1, wherein said food composition or oral and/or enteral preparation is for the prevention of allergic reactions in the gastrointestinal tract in humans or animals.

Patentansprüche

1. Verwendung einer einen transformierenden Wachstumsfaktor Beta 2 (TGF-β2) enthaltenden sauren Caseinfraktion, die aus Kuhmilch isoliert wurde, zur Herstellung einer Nahrungsmittel-Zusammensetzung oder einer oralen und/oder enteralen Zubereitung zur Modulierung der Expression des Haupthistokompatibilitätskomplexes der Klasse II (MHC-class II) durch intestinale Epithelzellen in Menschen oder Tieren, wobei die Nahrungsmittelzusammensetzung oder die orale und/oder enterale Zubereitung 0,1 bis 50 μg TGF-β2 pro g Trockensubstanz enthält.

2. Verwendung nach Anspruch 1, bei der die genannte Nahrungsmittelzusammensetzung oder die orale und/oder enterale Zubereitung zur Behandlung von entzündlichen Darmerkrankungen oder von Transplantat-Wirt-Reaktionen bei Menschen oder Tieren bestimmt ist.

3. Verwendung nach Anspruch 2, bei der die genannte entzündliche Darmerkrankung die Crohn Krankheit ist.

4. Verwendung nach Anspruch 1, bei der die genannte Nahrungsmittelzusammensetzung oder die orale und/oder enterale Zubereitung zur Verhinderung von Diarrhöe bei Menschen oder Tieren bestimmt ist.

5. Verwendung nach Anspruch 1, bei der die genannte Nahrungsmittelzusammensetzung oder die orale und/oder enterale Zubereitung zur Verhinderung von allergischen Reaktionen im Gastrointestinaltrakt bei Menschen oder Tieren bestimmt ist.

Revendications

1. Utilisation d'une fraction caillée à l'acide contenant le facteur de croissance transformant β2 (TGF-β2) isolée à partir du lait de vache pour la préparation d'une composition alimentaire ou d'une préparation à absorption orale et/ou entérique destinée à la modulation de l'expression des antigènes de classe II du complexe majeur d'histocompatibilité (Classe II du CMH) par les cellules épithéliales intestinales chez l'homme et les animaux, dans laquelle la composition alimentaire ou la préparation à absorption orale et/ou entérique contient de 0,1 à 50 µg de TGF-β2 par gramme de matières sèches.

2. Utilisation selon la revendication 1, dans laquelle ladite composition alimentaire ou préparation à absorption orale et/ou entérique est destinée au traitement des maladies inflammatoires de l'intestin ou les réactions du greffon contre l'hôte chez l'homme ou les animaux.

3. Utilisation selon la revendication 2, dans laquelle ladite maladie inflammatoire de l'intestin est la maladie de Crohn.

4. Utilisation selon la revendication 1, dans laquelle ladite composition alimentaire ou préparation à absorption orale et/ou entérique est destinée à la prévention de la diarrhée chez les nourrissons ou les animaux à peine sevrés.

5. Utilisation selon la revendication 1, dans laquelle ladite composition alimentaire ou préparation à absorption orale et/ou entérique est destinée à la prévention des réactions allergiques dans le tube gastro-intestinal chez l'homme ou les animaux.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 064 857 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
09.06.2004 Bulletin 2004/24

(51) Int Cl.[7]: **A23L 1/30**

(21) Application number: **00113787.6**

(22) Date of filing: **29.06.2000**

(54) **Method of producing compositions containing polyamines**

Verfahren zur Herstellung von Polyamin enthaltenden Zusammensetzungen

Méthode pour la production de compositions contenant des polyamines

(84) Designated Contracting States:
DE DK FR NL

(30) Priority: **30.06.1999 JP 18463799**

(43) Date of publication of application:
03.01.2001 Bulletin 2001/01

(73) Proprietor: **SNOW BRAND MILK PRODUCTS CO., LTD.**
Sapporo, Hokkaido 065-0043 (JP)

(72) Inventors:
- **Tanimoto, Yoshihiro No. 203,Yukijirushi Oaza Kawagoe-shi, Saitama-ken (JP)**
- **Yakabe, Takafumi Tsurugashima-shi, Saitama-ken (JP)**
- **Shimatani, Masaharu Sayama-shi, Saitama-ken (JP)**
- **Shigematsu, Akinori Kawagoe-shi, Saitama-ken (JP)**
- **Tomizawa, Akira Iruma-shi, Saitama-ken (JP)**

(74) Representative: **VOSSIUS & PARTNER**
Siebertstrasse 4
81675 München (DE)

(56) References cited:
EP-A- 0 729 710 **GB-A- 1 363 783**

- **DATABASE WPI Week 199503 Derwent Publications Ltd., London, GB; AN 1995-019175 XP002150136 & JP 06 305956 A (SNOW BRAND MILK PROD CO LTD), 1 November 1994 (1994-11-01)**
- **DATABASE WPI Week 199826 Derwent Publications Ltd., London, GB; AN 1998-289843 XP002150137 & JP 10 099048 A (SNOW BRAND MILK PROD CO LTD), 21 April 1998 (1998-04-21)**

EP 1 064 857 B1

Description

[0001] The present invention relates to methods of producing compositions containing polyamines.

[0002] The present invention relates to a method of producing compositions containing polyamines easily at low cost, in which milk or milk material is treated with an ultrafiltration (UF) membrane at a pH lower than 5.5 and polyamines are isolated and recovered from the resulting permeate. Furthermore, the present invention relates to methods of producing compositions containing polyamines which have a good flavor, contain high concentrations of spermidine and spermine and are highly utilizable as a food material.

[0003] Polyamine is a general term for a linear aliphatic hydrocarbon having two or more primary amino groups. Typical polyamines include putrescine, spermidine and spermine. Generally known physiological actions of polyamines are (1) action on cell growth, (2) stimulation of cell differentiation, (3) action as immunoessential factors, (4) antiallergic action, (5) stimulation of protein synthesis, (6) structural stabilization by an interaction with nucleic acids, (7) control of enzyme activities, and the like. Recently, there have been a number of reports that polyamines effectively stimulate the growth and differentiation of mucosal cells in the gastrointestinal

[0004] Examples of these reports are as follows:

[0005] O. Peulen et al., Arch. Physiol. Biochem., vol. 106, pp. 46-55, 1998; W.P. Deloyer et al., Arch. Physiol. Biochem., vol. 104, pp. 163-172, 1996; M. Kaouass et al., Dig. Dis, Sci., vol. 41, pp. 1434-1444, 1996; E. Harada et al., Comp. Biochem. Physiol., vol. 109A, pp. 667-673, 1994; G. Capano et al., J. Pediatr. Gastroenterol. Nutr., vol. 19, pp. 34-42, 1994; G.E. Wild et al., Biol. Neonate, vol. 63, pp. 246-257, 1993; Buts J. -P. et al., Digestive Diseases and Science, vol. 38, p. 1091, 1993; Dufour, C. et al., Gastroenterology, vol. 95, p. 112,1988.

[0006] The physiological effects of spermidine and spermine were studied and a stimulative action of polyamines on maturation of the gastrointestinal was revealed in these reports. Accordingly, in order to render to food products a stimulative effect on maturation of the gastrointestinal by adding polyamines, there was a need to produce polyamine-containing compositions having a good flavor and higher concentrations of spermidine or spermine.

[0007] Reported, examples of the use of polyamines in foods include a konnyaku (a food made from devil's-tongue starch) product in which spermine or spermidine is added to ameliorate the unique konnyaku flavor so as not to adversely affect the flavor of other ingredients cooked together (Japanese Patent Laid-open No. 38690/1994) and a polyamine-admixed nutritional composition in which polyamines are admixed to stimulate protein absorption for promoting satisfactory growth and maintaining good healthy conditions (Japanese Patent Laid-open No. 1994-305956). Examples of the use of polyamines as medicines include a method and ingestible compositions for the suppression of gastric acid secretion (Japanese Patent Laid-open No. 131914/1983) and immunoactivators (Japanese Patent Laid-open No. 98015/1984; Japanese Patent Laid Open No. 223514/1990).

[0008] Examples of conventional methods to produce polyamine compositions for the abovementioned purposes include a method of producing polyamines using milts as the raw material (Japanese Patent Laid-open No. 238094/1996), a method of producing polyamines using animal organs as the raw material (Japanese Patent Laid-open No. 206025/1997), and a method of producing polyamines from yeast cells and a nutritional polyamine-containing composition produced by the same method (Japanese Patent Laid-open No. 52291/1998). Since milts, animal organs and yeast cells are used as the raw material, polyamines produced by these methods smell of fish, animals and yeast and the flavor is not altogether agreeable. If milts are used as the raw material for producing a polyamine-containing composition, the material is highly viscous and difficult to handle, making purification inefficient.

[0009] In the abovementioned polyamine-admixed nutritional composition (Japanese Patent Laid-open No. 305956/1994), polyamines are isolated and extracted from milk materials from mammals using an ultrafiltration (UF) membrane or ion exchange resins. However, the yield ofpolyamine extraction is low and problems remain to be solved for practical use of this method.

[0010] An objective of the present invention is to provide a method of producing polyamine-containing compositions which have a good flavor and higher concentrations of spermidine and spermine, from milk or milk material.

[0011] In the course of an intensive study to develop a method of producing polyamine-containing compositions having higher concentrations of spermidine and spermine, the present inventors found that polyamine-containing compositions which have a good flavor and higher concentrations of spermidine and spermine can be produced by treating milk or milk material with an UF membrane at a pH lower than 5.5 and isolating and recovering polyamines from the resulting permeate to efficiently fractionate polyamines in the milk or milk material, and completed the present invention.

[0012] In the present invention, polyamine-containing compositions are produced by treating milk or milk material with an UF membrane at a pH lower than 5,5 and isolating and recovering polyamines from the resulting permeate. In this case, the pH has a great impact, such that the recovery of the polyamine-containing compositions is poor if the pH is higher than 5.5.

[0013] Examples of the milk to be used as a raw material in this invention include any milk of mammals, such as cow milk, goat milk and ewe milk. Examples ofthe milk material include any milk component isolated and/

or fractionated using an ion exchange method, membrane treatment, electrodialysis, or the like, such as acid casein, acid whey, cheese whey, whey protein concentrate (WPC), whey protein isolate (WPI), powdered skim milk, butter milk and cream.

[0014] The pH of the milk or milk material can be adjusted below 5.5 by adding acid to a solution of the milk or milk material or by adding microorganisms for microbial growth. The addition of the acid and microorganisms can be done simultaneously. Examples of the acid to be used include any inorganic and organic acid, such as lactic acid, citric acid, sulfuric acid, hydrochloric acid, acetic acid and phosphoric acid. Microorganisms can be any microorganisms, which can utilize and grow on milk components and produce organic acids, such as lactic acid bacteria, propionic acid bacteria and bifidobacteria. The presence of enzymes such as rennet should not cause any problem.

[0015] Thus, polyamine-containing compositions which have a good flavor and higher concentrations of spermidine and spermine can be produced by treating milk or milk material with an UF membrane at a pH lower than 5.5 and isolating and recovering polyamines.

[0016] Preferred embodiments will now be described in detail.

[0017] Polyamine-containing compositions can be produced by lowering the pH of milk or milk material to less than 5.5 with the addition of acid or by the growth of microorganisms, treating the acidified milk or milk materials with an UF membrane and isolating and recovering polyamines from the resulting permeate.

[0018] Examples of the UF membrane can be any membrane having a fractionation molecular weight of 1,000-100,000 including organic membranes, such as cellulose, cellulose acetate, polysulfone, polyamide, polyacrylonitrile, polyethylene tetrafluoride, polyester, polypropylene or the like, and inorganic membranes such as ceramics with aluminum, zirconium, titanium or the like, and glass (silica) or the like.

[0019] Steps such as a treatment with ion exchange resins, treatment with NF (nanofiltration), and electrodialysis can be used for the purification or desalting of the polyamine-containing solution. These steps can be used in combination to obtain further purified polyamine-containing compositions.

[0020] The treatment with ion exchange resins can be carried out such that milk or milk material is treated with an UF membrane at a pH below 5.5, after which the resulting permeate, with or without adjusting the pH by adding alkali such as sodium hydroxide and potassium hydroxide, is passed through a column filled with ion exchange resins to separate polyamines from contaminants such as amino acids, peptides, proteins and sugars. Any ion exchange resins having an ion exchange group, such as sulfonyl, sulfopropyl, phosphate, carboxymethyl, aminoethyl, diethylamino, quaternary aminoethyl and quaternary ammonium groups, can be used. Both cation exchange resins and anion exchange resins can be used.

[0021] If cation exchange resins are used, since polyamines are adsorbed onto cation exchange resins, the unadsorbed substances are first thoroughly removed, then the polyamines can be eluted and recovered using acidic solutions such as sulfuric acid or hydrochloric acid, or salt solutions such as sodium chloride. If anion exchange resins are used, polyamines can be recovered from the unabsorbed fraction since polyamines are not adsorbed onto anion exchange resins.

[0022] NF membranes can be used for desalting. Any NF membrane with a salt inhibition rate of 30-80% can be used.

[0023] Electrodialysis can be carried out by placing a polyamine-containing solution and a salt solution alternately in compartments sectioned by cation exchange membranes and anion exchange membranes. Desirable conditions for electrodialysis are a primary current density of 0.5-15 A/dm^2 and a voltage of 0.1-1.5 V/bath.

[0024] The polyamine-containing compositions thus obtained can be used as solutions without further processing or spray-dried or lyophilized to obtain powdered products.

[0025] The polyamine-containing compositions thus obtained can be used as medicaments and medicinal nutrient compositions such as enteral nutrients, nutrient compositions for infants, such as powdered milk for infants and baby foods, and as raw materials for nutritionally fortified foods or general food products

[0026] The present invention will be explained in more detail with reference to the following examples and comparative examples.

[0027] Analysis of polyamine contents in the examples and comparative examples was carried out by the method of Kawakami et al. (Japanese Journal of Pediatric Gastroenterology and Nutrition, vol. 9, pp. 115-121, 1995).

[Example 1]

[0028] A composition containing polyamines derived from milk was produced. Raw milk (cow's milk) was centrifuged (2,000 g, 4C, 10 minutes) to prepare skimmed milk, To this skimmed milk, 1N hydrochloric acid was added to adjust the pH to 4.6, and the resulting milk was centrifuged (35,000 g, 4C, 20 minutes) to remove casein. This acid whey thus obtained was treated with an UF membrane with a fractionation molecular weight of 8,000 to recover an UF permeate. This permeate was passed through a column filled with cation exchange resins (Dowex 50WX8 (H^+ form)) to allow polyamines to adsorb onto the cation exchange resins, after which the column was thoroughly washed with a 0.5 M sodium chloride solution to remove impurities, then the adsorbed polyamines were eluted with 6N hydrochloric acid. This eluate was neutralized with the addition of a sodium hydroxide solution, electrodialyzed for desalting,

and lyophilized to obtain a polyamine-containing composition.

[0029] Thus, 528 mg of a polyamine-containing composition were obtained from 1,000 L of acid whey. The sum of the percentage of spermidine and spermine in the total polyamines was 97.5%.

[Example 2]

[0030] A polyamine-containing composition was produced using cottage cheese whey as a milk material. Whey (pH 4.3) produced during cottage cheese manufacturing was treated with an UF membrane (having a fractionation molecular weight of 5,000) to recover a permeate. This permeate was passed through a column filled with cation exchange resins (Dowex 50WX8 (H^+ form)) to allow polyamines to adsorb onto the cation exchange resins, after which the column was thoroughly washed with a 0.6 M sodium chloride solution to remove impurities, then the adsorbed polyamines were eluted with 6N hydrochloric acid. This eluate was neutralized with the addition of a sodium hydroxide solution, electrodialyzed for desalting, and lyophilized to obtain a polyamine-containing composition.

[0031] Thus, 426 mg of a polyamine-containing composition were obtained from 1,000 L of cottage cheese whey. The sum of the percentage of spermidine and spermine in the total polyamines was 98.5%.

[Comparative Example 1]

[0032] A polyamine-containing composition was produced using Gouda cheese whey as a milk material. Whey (pH 6.2) produced during Gouda cheese manufacturing was treated with an UF membrane (having a fractionation molecular weight of 5,000) to recover a permeate. This permeate was passed through a column filled with cation exchange resins (Dowex 50WX8 (H^+ form)) to allow polyamines to adsorb onto the cation exchange resins, after which the column was thoroughly washed with a 0.6 M sodium chloride solution to remove impurities, then the adsorbed polyamines were eluted with 6N hydrochloric acid. This eluate was neutralized with the addition of a sodium hydroxide solution, electrodialyzed for desalting, and lyophilized to obtain a polyamine-containing composition.

[0033] Thus, 515 mg of a polyamine-containing composition were obtained from 1,000 L of Gouda cheese whey. However, neither spermidine nor spermine was detected in this polyamine-containing composition.

[Test Example 1]

[0034] Polyamine-containing compositions each prepared using milts, animal organs, yeast cells and milk as a raw material were added to a food product, and the effect of the addition on the quality of the food product was evaluated by a sensory evaluation. Polyamine-containing compositions were prepared from milts by the method of Japanese Patent Laid-open No. 238094/1996, from animal organs by the method of Japanese Patent Laid-open No. 206025/1997, and from yeast cells by the method of Japanese Patent Laid-open No. 52291/1998, respectively. The polyamine-containing compositions prepared in Examples 1 and 2 were used as polyamines prepared from milk or milk material. The polyamine-containing compositions obtained from different raw materials were added to whole fat powdered milk so as to adjust the concentration of the sum of spermidine and spermine to 10 mg/100 g to prepare polyamine-added compositions for a sensory evaluation. The evaluation was for "odor" and "taste."

[0035] The sensory evaluation was carried out by 10 male and 10 female trained panelists using a scoring method. Results of the sensory test are shown as averages of the scores given by the 20 panelists, according to the following scales for evaluation.

[0036] Results are shown in Table 1. The scales for evaluation are as follows:

Odor - 5: No abnormal odor; 4: faintly abnormal odor; 3: slightly abnormal odor; 2: abnormal odor; and 1: strongly abnormal odor.
Taste - 5: No abnormal taste; 4: faintly abnormal taste; 3: slightly abnormal taste; 2: abnormal taste; and 1: strongly abnormal taste.

I. [TABLE 1]

Raw material	Odor	Taste
Salmon milts	1.9	1.1
Caw pancreas	1.1	1.0
Pig pancreas	1.5	1.4
Yeast cells (*Candida utilis*)	4.0	4.6
Acid whey	4.8	4.8
Cottage cheese whey	4.7	4.8
Control (whole milk powder)	4.9	4.9

[0037] The present invention enables polyamine-containing compositions to be easily obtained at low cost by treating milk or milk material with an ultrafiltration (UF) membrane at a pH below 5.5 and isolating and recovering polyamines from the resulting permeate.

[0038] Since the polyamine-containing compositions obtained in the present invention are characterized by a good flavor and high contents of spermidine and spermine, they can be effectively used as medicaments and medicinal nutrient compositions, such as enteral nutrients, nutrient compositions for infants, such as infant powdered milk and baby foods, and as raw materials for nutritionally fortified food products and general food products.

Claims

1. A method of producing polyamine-containing compositions which includes treating milk or milk material with an ultrafiltration (UF) membrane at a pH lower than 5.5 and isolating and recovering polyamines from the resulting permeate.

2. A method of producing polyamine-containing compositions as claimed in claim 1 wherein inorganic acids or organic acids, and/or microorganism, which utilize milk components for growth to produce organic acids, are added to lower the pH of the milk or milk material to 5.5.

3. A method of producing polyamine-containing compositions as claimed in claim 1 or 2, wherein polyamines are isolated by one or more steps of an ion exchange process, membrane fractionation process and electrodialysis.

4. A method of producing polyamine-containing compositions as claimed in claim 2 or 3, wherein said inorganic acids of organic acids are selected from lactic acid, citric acid, sulfuric acid, hydrochloric acid, acetic acid and phosphoric acid.

5. A method of producing polyamine-containing compositions as claimed in any of claims 2 to 4, wherein said microorganisms are selected from lactic acid bacteria, propionic acid bacteria and bifidobacteria.

Patentansprüche

1. Verfahren zur Herstellung von Polyamin-enthaltenden Zusammensetzungen, welches die Behandlung von Milch oder Milchmaterial mit einer Ultrafiltrations(UF)-Membran bei einem pH-Wert von weniger als 5,5 und die Isolation und Gewinnung von Polyaminen aus dem resultierenden Permeat einschließt.

2. Verfahren zur Herstellung von Polyamin-enthaltenden Zusammensetzungen gemäß Anspruch 1, wobei anorganische oder organische Säuren und/oder Mikroorganismen, welche Milchkomponenten zum Wachstum oder zur Produktion von organischen Säuren nutzen, zugegeben werden, um den pH-Wert der Milch oder des Milchmaterials auf 5,5 zu verringern.

3. Verfahren zur Herstellung von Polyamin-enthaltenden Zusammensetzungen gemäß Anspruch 1 oder 2, wobei die Polyamine durch einen oder mehrere Schritte, ausgewählt aus einem Ionenaustauschverfahren, einem Membranfraktionierungsverfahren und einer Elektrodialyse isoliert werden.

4. Verfahren zur Herstellung von Polyamin-enthaltenden Zusammensetzungen gemäß Anspruch 2 oder 3, wobei die anorganischen oder organischen Säuren ausgewählt sind aus Milchsäure, Citronensäure, Schwefelsäure, Salzsäure, Essigsäure und Phosphorsäure.

5. Verfahren zur Herstellung von Polyamin-enthaltenden Zusammensetzungen gemäß einem der Ansprüche 2 bis 4, wobei die Mikroorganismen ausgewählt sind aus Milchsäurebakterien, Propionsäurebakterien und Bifidobakterien.

Revendications

1. Procédé de production de compositions contenant des polyamines, qui inclut le traitement de lait ou de matière laitière avec une membrane d'ultrafiltration (UF) à un pH inférieur à 5,5 et l'isolement et la récupération des polyamines à partir du perméat obtenu.

2. Procédé de production de compositions contenant des polyamines selon la revendication 1, dans lequel des acides minéraux ou des acides organiques, et/ou des micro-organismes qui utilisent des constituants laitiers pour se développer et produire des acides organiques, sont ajoutés pour abaisser le pH du lait ou de la matière laitière à 5,5.

3. Procédé de production de compositions contenant des polyamines selon la revendication 1 ou 2, dans lequel les polyamines sont isolées par une ou plusieurs étapes parmi un procédé d'échange d'ions, un procédé de fractionnement par membrane et l'électrodialyse.

4. Procédé de production de compositions contenant des polyamines selon la revendication 2 ou 3, dans lequel lesdits acides minéraux ou acides organiques sont choisis parmi l'acide lactique, l'acide citrique, l'acide sulfurique, l'acide chlorhydrique l'acide acétique et l'acide phosphorique.

5. Procédé de production de compositions contenant des polyamines selon l'une quelconque des revendications 2 à 4, dans lequel lesdits micro-organismes sont choisis parmi les bactéries lactiques, les bactéries propioniques et les bifidobactéries.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 August 2001 (16.08.2001)

PCT

(10) International Publication Number
WO 01/58283 A1

(51) International Patent Classification[7]: A23L 1/305, A61K 31/195

(21) International Application Number: PCT/NL01/00104

(22) International Filing Date: 8 February 2001 (08.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1014380 14 February 2000 (14.02.2000) NL

(71) Applicant *(for all designated States except US)*: **FRIESLAND BRANDS B.V.** [NL/NL]; P.O. Box 226, NL-8901 MA Leeuwarden (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **VAN LEEUWEN, Paulus, Aluisius, Marie** [NL/NL]; Simon Carmiggeltlaan 1, NL-1187 VN Amstelveen (NL). **HOUDIJK, Alexander, Petrus, Johannes** [NL/NL]; Van der Ghiessenstraat 25, NL-1181 RS Amstelveen (NL). **GLAS, Cornelis** [NL/NL]; Woelwijk 3, NL-9255 KE Tietjerk (NL).

(74) Agent: **JORRITSMA, Ruurd**; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/58283 A1

(54) Title: USE OF GLUTAMATE AND/OR A GLUTAMATE PRECURSOR FOR THE PREPARATION OF A NUTRITIONAL OR PHARMACEUTICAL PREPARATION FOR THE TREATMENT OR PREVENTION OF HYPERPERMEABILITY OR UNDESIRED PERMEABILITY OF THE INTESTINAL WALL

(57) Abstract: The present invention relates to the use of glutamic acid for the preparation of a nutritional preparation that is intended for use for the treatment or prevention of excess or undesired permeability of the intestinal wall. In particular, according to the invention the glutamic acid is used in a nutritional preparation, such as a baby food or an enteral food. Examples of conditions where glutamic acid is used are: food allergy, internal drug allergy, sepsis, low blood flow through the intestines, ICU patients, surgical interventions, malnutrition or intestinal maturation of newborn babies.

USE OF GLUTAMATE AND/OR A GLUTAMATE PRECURSOR FOR THE PREPARATION OF A NUTRITIONAL OR PHARMACEUTICAL PREPATION FOR THE TREATMENT OR PREVENTION OF HYPERPERMEABILITY OR UNDESIRED PERMEABILITY OF THE INTESTINAL WALL

The invention relates to the preparation of a nutritional preparation that is suitable for use in the case of conditions associated with an increased permeability of the intestinal wall.

The intestinal epithelium acts as a selective barrier which allows the absorption of nutrients but restricts the passage of microorganisms and undesired macromolecules. Maintaining this barrier is considered to be important in order to protect the host against the migration of pathogenic microorganisms from the intestines to the bloodstream. It is assumed that the increase in the permeability of the intestines is associated with damage to the paracellular transport system of the intestinal mucosa, as a result of which translocation of endotoxins and (pathogenic) bacteria can occur. As a result of the damage to the intestinal mucosa it is also possible for absorption of macromolecules to occur, which are then able to initiate allergic reactions.

An increase in the permeability of the intestinal wall has been detected in clinical conditions associated with damage to the intestinal mucosa barrier, such as endotoxaemia, sepsis, multiple trauma, malnutrition, major surgical interventions, parenteral nutrition and burns. An increase in the permeability to larger molecules, such as proteins, has been found in the newborn, but also occurs in healthy people if they are allergic to food products.

It is known that glutamine is able to lower the macromolecular hyperpermeability of intestinal cells which is induced by phorbol 12,13-dibutyrate (Kouznetsova et al. *J. Parenteral Enteral Nutrition,* **23** (1999) 136-139). A disadvantage of glutamine, however, is that it is not stable at room temperature, which renders it unsuitable for (non-chilled) foods with a long shelf life. Moreover, glutamine has poor solubility.

Instead of glutamine, specific products based on peptides, mainly di- and tripeptides, are used. These peptides are frequently prepared from glutamine-rich vegetable proteins, such as, in particular, wheat protein, in which preparation method, following enzymatic conversion, fractionation technology is used in order to obtain the specific peptide fraction as the main fraction. Examples thereof can be found in JP 05236909 and JP 08157385. Such a preparation of these peptides is expensive and complex. Moreover, a product obtained from wheat protein can be problematic for some patients, such as coeliac patients.

It has now been found that too high a permeability of the intestinal wall can be effectively treated or prevented by the administration of a suitable quantity of glutamate and/or a glutamate precursor, preferably in a nutritional preparation. The invention therefore relates to a nutritional or pharmaceutical preparation containing glutamate and/or a glutamate precursor, in particular for such a use, as described in more detail in the appended claims.

From the state of the art, for instance US 5,366,723 it is known to use a combination of glutamic acid, aspartic acid and cystein in decreasing the toxicity of platinum compounds in the treatment of cancer. One of the activities mentioned is regeneration of the intestinal mucosa. However, regeneration of the intestinal mucosa relates to a different phenomena than permeability of the intestine which is in particular associated with integrity of the intestine or the paracellular transport via the intestinal mucosa.

Here a nutritional preparation is understood to be a composition that contains food constituents (at least one), such as proteins, carbohydrates, fats, vitamins, minerals and the like. Preferably the composition contains more than one food constituent and preferably it contains all necessary food constituents. It can therefore be a food supplement or a complete food or a food drug ("neutraceuticum").

The nutritional preparation of the invention can be an infant formula or children's food or an enteral (functional/clinical/problem solving) food.

Substances that are known to have a beneficial effect on the intestinal function (permeability) can also be added. In particular, one or more polyamines such as spermine, spermadine or putrescine or one or more polyamine precursors, in particular ornithin and arginin can be used. Such polyamines are, for example, described in Dorhout et al., *Br. J. Nutrition,* 1997, 639-654 and in a report of a seminar held on 29 June to 2 July 1999 in Glasgow, published in Proceedings of the Nutrition Society, Vol. 59 (Issue 1), 2000, 81-86. Polyamines or the precursors thereof can have a beneficial contribution in e.g. postnatal intestinal maturation, permeability of the intestine to macromolecules (allergy), the translocation of bacteria, etc.

The preparation contains preferably 0.05 to 10 g, more preferably 0.2 to 4 g free glutamate per 100 g of the nutritional preparation (dry weight). The polyamines are preferably present in an amount of 1 to 10 mg per 100 g of the nutritional preparation (dry weight).

The glutamate is preferably incorporated in a nutritional preparation alongside proteins or peptides, such as lactic or vegetable proteins. The nutritional preparation contains in particular lactic proteins or hydrolysates obtained therefrom. Lactic proteins comprise casein, whey proteins and lactoferrin.

Carbohydrates are understood to be digestible carbohydrates, such as glucose, lactose, maltose and sucrose, and digestible oligosaccharides and polysaccharides, such as maltodextrins, amylopectins and starch, as well as non-digestible carbohydrates (food fibres) such as galacto-oligosaccharides or fructo-oligosaccharides (inulin), vegetable and animal and microbial gums, such as carob bean flour and gum arabic. Fats comprise vegetable and animal fats, fats with medium length chains (C_8-C_{12}) (MCT), fats with unsaturated long chains (such as γ-linolenic acid, arachidonic acid, eicosapentaenoic acid and docosahexaenic acid).

The nutritional preparation according to the invention can also contain glutamine or an equivalent thereof. Glutamine equivalents are known to those skilled in the art. Examples of these are the abovementioned dipeptides and tripeptides. If the nutritional preparation is a food for babies or toddlers, the weight ratio of glutamic acid : glutamine from the free amino acids is greater than 1 : 1, in particular greater than 5 : 1 and very particularly greater than 25 : 1.

Here a glutamate precursor is meant to include glutamic acid or alfa-keto glutaric acid, a biochemical precursor. Glutamate can be in the form of a physiologically acceptable glutamate salt (for example the sodium, potassium, calcium or magnesium salt). As a source of glutamate protein hydrolysates can be used or freeze dried cultures of (lactic acid) bacteria (probiotics) which contain glutamate as a protecting agent. An example of such a lactic acid culture is *Lactobacillus Reuteri*, obtainable from Biogaia, originating from human milk.

Free glutamic acid is understood to be glutamic acid or a salt thereof that is not bound in protein or peptide and that has either been added or is present in the free amino acid fraction of a protein or hydrolysed protein (hydrolysed proteins usually contain 10 - 20% free amino acids) or is present as a protecting agent in a probiotic lactic acid culture.

The preparations of the invention are preferably combined with suitable prebiotics and probiotics, which have a beneficial effect on the intestinal flora. The prebiotics comprise short or long chain oligosaccharides, in particular galacto-oligosaccharides and

fructo-oligosaccharides, branched oligosaccharides, sialyloligosaccharide, nucleotides, protein hydrolysates, sialic acid rich milk products or derivatives thereof, etc.

The nutritional preparation to be prepared according to the invention can be used in the treatment of all conditions where hyperpermeability of the intestinal wall is concerned. Examples of these are food allergy, allergy to internal drugs, sepsis and similar clinical conditions, translocation of pathogenic bacteria through the intestinal wall, endotoxaemia, viral diarrhoea, low intestinal blood flow, IC patients, patients after surgical interventions or with major burns, parenteral nutrition and undernutrition. It can also be used in the case of intestinal maturation of newborn babies, reduction of abnormal crying in children or the treatment of hyperactivity (Attention Deficit Hyperactivity Disorder, ADHD).

Example 1

The macromolecular permeability of the intestinal epithelium is controlled by the passage through intercellular tight junctions in the paracellular channels. Opening of these tight junctions is controlled by the epithelial cells in response to various intercellular mediators, such as Ca, cyclic AMP, G proteins and protein kinase c. The human intestinal cell line HT-29CL.19A is becoming increasingly more important for studying this paracellular permeability in vitro. See also the abovementioned article in *J. Parenteral Enteral Nutrition*, that is incorporated herein by reference.

For the present examples, confluent monolayers of HT-29CL.19A cells were cultured on permeability filters. After 14 - 17 days the cells were allowed to grow for a further two days without glutamine in the medium. The transepithelial permeability from apical to basolateral was determined for horseradish peroxidase (HRP) with the aid of an enzyme assay. Phorbol 12,13-dibutyrate (PDB, 1 mmol/l) was used to increase the permeability. The effect of glutamine, glutamate and the g-glutamyl transferase inhibitor acivicin was investigated. All agents were added to the apical compartment.

It was found that PDB increases the HRP flux 3-fold compared with the control after 150-279 min stimulation ($p < 0.001$). Glutamine reduces this hyperpermeability appreciably. Glutamate (0.6 mmol/l) had the same effect ($p < 0.001$). Acivicin prevented the glutamine-mediated reduction in the hyperpermeability induced by PDB. This effect did not occur in the presence of glutamate.

It can be seen from this experiment that glutamate reduces the macromolecular

hyperpermeability in HT-29CL.19A cells.

Example 2

A complete, pulverulent, glutamic acid-containing baby food for premature children was prepared which had the following composition per 100 g powder - dry matter:

desalinated whey, solids	39.2 g
vegetable fats	26.4 g
lactose	17.9 g
skimmed milk, solids	13.4 g
glucose syrup	0.90 g
soy lecithin	0.16 g
glutamic acid	0.5 g
L-arginine	0.05 g
taurine	0.04 g
L-Tryptophane	0.02 g
nucleotides	0.03 g
microminerals and vitamins	1.4 g
casein/whey protein ratio	40/60
% crude protein	10.8
% free glutamic acid	0.5

A fluid composition that contains approximately 15% solids can be prepared from such a powder. Approximately 175 ml of the fluid composition is administered per kg body weight per day.

Example 3

A food was prepared as in example 2, with the exception that instead of 0.7 g lactose 0.7 g galacto-oligosaccharides were incorporated per 100 g powder.

Example 4

A complete, pulverulent, glutamic acid-containing baby food for children with an

allergy was prepared which had t ıe following composition per 100 g powder - dry matter:

hydrolysed casein	13.5 g
vegetable fat	27 g
glucose syrup	58.05 g
taurine	0.04 g
L-carnitine	0.01 g
microminerals and vitamins	1.4 g
% crude protein	12
% free ornithin	0.01
% free glutamic acid	0.3

Example 5

A food was prepared according to example 4, with the exception that 0.25 g arginin and 0.005 g spermine and spermidine was incorporated instead of 0.255 g glucose syrup.

Example 6

A pulverent food for young children was prepared to limit excessive crying which contained per 100 gram:

lactic protein	11 g
fat	27 g
lactose	56 g
nucleotides	0.03 g
glutamic acid	0.45 g
minerals, vitamins, probiotic	3.02 g
water	2.5 g

ratio whey protein : casein : casein hydrolysate 40:30:30

L. Reuteri (Biogaia)	1×10^8
% free glutamic acid	0.5

Example 7

A pulverent food for older children with multiple functional properties was prepared, containing per 100 grams:

lactic protein	22.1 g
fat	18.4 g
lactose	39 g
sucrose	10 g
alfa-ketoglutarate	0.1 g
fructo-oligosaccharide (inulin)	3 g
nucleotides	0.05 g
minerals, vitamins, probiotics	4.85 g
water	2.5 g

L. Reuteri (Biogaia)	1×10^8

casein/whey protein ratio	75:25

goat's milk protein (comprising human-milk like sialyloligosaccharides) : 20 % of the lactic protein

% alfa-ketoglutarate	0.1
% free glutamic acid	0.05
% free glutamic acid equivalents	0.15

Example 8

A pulverent food for children having hyperactivity syndrome (ADHD) was prepared containing, compared to example 7, a casein/casein hydrolysate ratio in the lactic protein fraction of 40:60.

The product contains per 100 g

L. Reuteri	1×10^8
% free glutamic acid equivalents	0.45

Example 9

A problem-solving, fluid, glutamic acid-containing enteral food based on caseinate and glutamine-rich vegetable hydrolysed protein (30% glutamine) was prepared which had the following composition per 100 g:

caseinate	5.2 g
glutamine-rich hydrolysed protein	1.0 g
glutamic acid	0.6 g
arginine	0.2 g
fats	3.4 g
carbohydrates	9.5 g
minerals and vitamins	0.4 g
lecithin	0.1 g
water	79.6 g

% crude protein	6.3 g
% free arginin	0.2 g
% glutamin	0.3 g
% free glutamic acid	0.6 g

Claims

1. Use of glutamate and/or a glutamate precursor for the preparation of a nutritional or pharmaceutical preparation for the treatment or prevention of hyperpermeability or undesired permeability of the intestinal wall.

2. Use according to claim 1, wherein the nutritional preparation is an infant formula or children's food.

3. Use according to claim 1 or 2, wherein the nutritional preparation is an enteral food or a food supplement.

4. Use according to any one of claims 1 to 3, wherein the nutritional preparation also contains lactic proteins or hydrolysed lactic proteins.

5. Use according to any one of claims 1 to 4, wherein the nutritional preparation also contains vegetable proteins or hydrolysed products thereof.

6. Use according to any one of claims 1 to 5, wherein hydrolysed protein is used as the source of glutamate.

7. Use according to any one of claims 1 to 6, wherein the glutamate precursor is glutamic acid or alfa-keto glutaric acid.

8. Use according to any one of the preceding claims, wherein the nutritional preparation also contains glutamine or an equivalent thereof.

9. Use according to claim 8, wherein the weight ratio of glutamic acid : glutamine in the free amino acid fraction is greater than 1 : 1, in particular greater than 5 : 1 and preferentially greater than 25 : 1.

10. Use according to any one of the preceding claims, wherein the preparation further

contains one or more polyamines. in particular spermine, spermidine or putrescine and/or one or more polyamine precursors, in particular ornithin and arginin.

11. Use according to any one of the preceding claims, wherein the nutritional preparation contains 0.05 - 10 g, preferably 0.2 - 4 g free glutamate per 100 g nutritional preparation (dry weight).

12. Use according to any one of the preceding claims, wherein the nutritional preparation further contains one or more prebiotics, preferably selected from the group consisting of protein hydrolysates, nucleotides, galacto-oligosaccharides, fructo-oligosaccharides, branched oligosaccharides and sialyloligosaccharides or equivalents thereof.

13. Use according to any one of the preceding claims, wherein the nutritional preparation contains freeze dried probiotics, in particular freeze dried *Lactobacillus Reuteri* as the source of glutamate.

14. Use according to any one of the preceding claims, in the case of deterioration of the mucosal barrier, intestinal dysfunction or injury, suboptimal intestinal wall maturation of new-borns, undernutrition, suboptimal intestinal blood flow, allergy, sepsis, translocation of pathogenic bacteria through the intestinal wall, endotoxaemia, viral diarrhoea, regularly crying children, hyperactive children, IC patients, patients after surgery or major burns.

15. Preparation containing free glutamate and/or a glutamate precursor and polyamines, in particular spermine, spermidine or putrescine and/or one or more polyamine precursors, in particular ornithin and arginin.

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/NL 01/00104

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23L1/305 A61K31/195

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAUL FRANCIS ET AL: "Functional and metabolic changes in intestinal mucosa of rats after enteral administration of ornithine alpha-ketoglutarate salt." JOURNAL OF PARENTERAL AND ENTERAL NUTRITION, vol. 19, no. 2, 1995, pages 145-150, XP001001310 ISSN: 0148-6071 page 145; table 1 page 146 --- -/--	1,3,7,8, 10,12, 14,15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 May 2001	28/05/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Heezius, A

4

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 01/00104

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EWTUSHIK A L ET AL: "Performance and intestinal development of piglets receiving diets supplemented with selected amino acids or polyamines." CANADIAN JOURNAL OF ANIMAL SCIENCE, vol. 77, no. 4, December 1997 (1997-12), pages 741-742, XP001001415 1997 Annual Meeting of the Canadian Society of Animal Science ISSN: 0008-3984 the whole document	1,3,5, 11,12, 14,15
X	DATABASE WPI Section Ch, Week 199342 Derwent Publications Ltd., London, GB; Class B04, AN 1993-330544 XP002148350 & JP 05 236909 A (SNOW BRAND MILK PROD CO) cited in the application abstract	1,3,5-8, 12,14,15
X	PATENT ABSTRACTS OF JAPAN vol. 1996, no. 10 & JP 08 157385 A (KIRIN BREWERY) cited in the application abstract	1,3,5-8, 12,14,15
X	US 5 366 723 A (TULOK ISTVAN) 22 November 1994 (1994-11-22) claims 7,10	1,3
A	US 5 981 590 A (GEWOLB IRA H ET AL) 9 November 1999 (1999-11-09) column 3, line 21 - line 29 claims 1,3,5,14	1-15

4

INTERNATIONAL SEARCH REPORT

Information on patent family members

In. ational Application No
PCT/NL 01/00104

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 05236909 A	17-09-1993	JP 2524551 B	14-08-1996
JP 08157385 A	18-06-1996	NONE	
US 5366723 A	22-11-1994	NONE	
US 5981590 A	09-11-1999	NONE	